Eight commercial lipase preparations were examined for the ability to hydrolyze phosphatidylcholine (PC) in hexane solutions. Only the enzymes from *Humicola lanuginosa*, *Rhizopus delemar* and *Candida rugosa* displayed appreciable activity. Solvent polarity was the largest single factor affecting activity. The *H. lanuginosa* sample was most active in polar solvents. The *R. delemar* preparation was most active in polar (2-hexanone) and nonpolar (decane) solvents and least active in solvents of intermediate polarity (hexane). The solvent dependence of the activity of the *C. rugosa* enzyme varied with the ratio of substrate to enzyme. Different degrees of activity were retained by the three enzymes after passive immobilization on Celite, controlled-pore glass, polypropylene and Amberlite XAD-7 resins. No single resin yielded the best retained activity for all three preparations. When examined in 2-octanone, hexane and isooctane, the Celite-immobilized *R. delemar* and *H. lanuginosa* enzymes exhibited highest activity in 2-octanone, while immobilized *C. rugosa* was most active in isooctane. The water content at which maximum activity was observed was relatively independent of solvent polarity and the amount of catalyst but was proportional to the amount of PC in the reaction. The retention of activity by immobilized *Rhizomucor miehei* lipase (Lipozyme) during multiple hydrolytic cycles required a reduction in the water content of the system below that yielding optimal activity in a single cycle.

KEY WORDS: Enzymatic hydrolysis, lipase, organic solvent, phosphatidylcholine, phospholipid.

Phospholipids (PLs) are of interest in the areas of both basic and applied science for a variety of reasons. Their biochemical functions are diverse and significant. As the major component of biological membranes, they play significant roles in conferring membrane integrity and function. In addition, they are participants in cellular communication and inflammation. Due to their amphipathic nature, PLs are efficient emulsifiers and have been developed for applications in the food, cosmetic and pharmaceutical sectors. They are also produced in substantial amounts as by-products of the isolation of other biological materials, particularly in the production of vegetable oils.

An understanding of the biological roles of PLs and a realization of their full potential in applied science and technology require the development of methods for their characterization and modification. Enzymes are attractive catalysts for these transformations due to their substrate and product specificities and their abilities to operate under ambient conditions. Various phospholipases hydrolyze the fatty acyl and phosphoryl ester bonds of PLs. However, there is renewed interest in the use of lipases to conduct some of these reactions. Not only do lipases generally include PLs within their substrate ranges (1–5); they are also commercially available from a much wider range of organisms and have been more thoroughly developed as applied catalysts than have the phospholipases.

Under appropriate conditions, i.e., in organic solvents containing limited amounts of water, lipases synthesize ester bonds. This phenomenon has been applied to alter the fatty acid composition of PLs (6–11). Lipases have also been employed in PL hydrolysis, for example, in the preparation of PLs and lysophospholipids of defined structure (12–14) and for the removal of contaminating PL from preparations of plasmalogens (15,16).

These hydrolytic reactions were conducted in aqueous solutions. However, PL has limited solubility in water, and the resulting mixtures can be very viscous. To capitalize on the higher solubilities and more homogenous solutions that result from the use of organic solvents in working with PLs, we recently examined the hydrolysis of phosphatidylcholine (PC) by an immobilized lipase in organic solvents (17). This study demonstrated the feasibility of enzymatic PL hydrolysis in solvents and examined several parameters that affected the efficiency of the reaction. In the present work, we have expanded this investigation to include other readily available lipases, examining their abilities to hydrolyze PC and the effects of various reaction parameters, including immobilization of the enzymes on insoluble carriers, on this reaction.

EXPERIMENTAL PROCEDURES

Materials. L-α-PC, (>99%, from soybeans) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Lipase preparations were the generous gifts of Amano International Enzyme Co., Inc. (Troy, VA). Lipozyme IM20 was obtained from NOVO Biolabs (Danbury, CT). Celite 545 diatomaceous earth was purchased from Fisher Scientific (Fair Lawn, NJ). Controlled-pore glass, 30/45 Mesh, 375A pore size, was obtained from Corning BIOsystems (Corning, NY). EP100 polypropylene powder was the product of AKZO (Obernburg, Germany). Amberlite XAD-7 was obtained from Sigma Chemical Co. (St. Louis, MO). 2-Hexanone, 2-butanone, isooctane and decane were the products of Aldrich Chemical Company, Inc. (Milwaukee, WI). Burdick and Jackson Brand hexane and isopropanol were purchased from Baxter (Muskegon, MI). Solvents were saturated at room temperature with distilled, deionized water before use.

Biochemical methods. Lipolytic activities in water were determined using a pH-stat method and a continuous titrating pH meter, with emulsified olive oil as the substrate (18). Protein contents of the lipase preparations were determined according to Bradford (19) with the Bio-Rad (Richmond, CA) Protein Assay Kit, with bovine-γ-globulin as the standard.

PC hydrolysis reactions. The standard reaction mixture contained 331 mg PC, enzyme, water and 8 mL of solvent. Water-saturated hexane was the solvent unless otherwise noted. Reactions were conducted for 15 h at 42°C in 20 × 150 mm screw-cap test tubes shaken at 300 rpm in an orbital shaker.

Analysis of hydrolysates. The degree of PC hydrolysis was determined by high-performance liquid chromatography (HPLC) as previously described (17). Hydrolysates were diluted 75-fold in hexane/isopropanol (2:3, vol/vol), and residual PC was quantitated on a Hewlett-Packard 1050 Chromatography System (Hewlett-Packard, Valley Forge, PA) with a 3 × 100 mm LiChrosorb DIOL column (Chrompack Inc., Raritan, NJ). The mobile phase was hexane/isopropanol/water (40:53.5:6.5, vol/vol/vol). Analyte was detected by means of a mass-based detector (ELSD IIA; Varex, Burtonsville, MD), and the amount of PC in each sample was determined by reference to a response curve generated with pure soybean PC. The hydrolysis of PC was calculated according to Equation 1:

$$\% \text{ PC hydrolysis} = (\text{original PC} - \text{remaining PC})/(\text{original PC}) \times 100 \quad [1]$$

All data were corrected for nonenzymatic hydrolysis, which was less than 5%.

Experimental design. In studying the abilities of the free enzymes to hydrolyze PC, a Central Composite Response Surface Design (20) was employed to coordinately investigate the effects and interactions of solvent polarity and of the concentrations of water, PC and amount of catalyst on the hydrolytic activities of the nonimmobilized enzymes at 42°C. The experimental settings of these variables are listed in Table 1. The centerpoint values were determined in preliminary experiments, designed to identify reaction conditions that yielded moderate hydrolytic activity. Hexane was chosen as the centerpoint solvent because of its acceptance in the fats and oils industry and in enzyme biotechnology. Solvent polarities were expressed and ranked in terms of log *P*, the logarithm of the partition coefficient in an octanol/water two-phase system (21). The greater the log *P*, the lower the polarity. For each enzyme, 40 hydrolytic reactions were conducted, including 12 replications at the midpoint. Reaction volumes were 8 mL. The amounts of enzyme and the lengths of incubation were chosen to keep the degree of hydrolysis below 90%. Reaction times were: *Humicola lanuginosa*, 1 h; *Rhizopus delemar*, 6 h; *Candida rugosa*, 6 h.

Enzyme immobilization. The enzymes (*H. lanuginosa*, 0.5 g; *R. delemar*, 0.4 g; *C. rugosa*, 2.0 g) were dissolved in approximately 3 mL of 20 mM sodium phosphate buffer (pH 7.5 for the *H. lanuginosa* and *C. rugosa* enzymes, pH 6.0 for the *R. delemar* lipase) at room temperature in glass Petri dishes (9-cm diameter). One gram (air-dried weight) of the support material was thoroughly mixed into this solution, and the water was evaporated under a stream of nitrogen (17–20 h). The residual material, which appeared completely dry, was stored in a screw-capped bottle at 4°C. Resins were used as received, except for the Amberlite, which was washed once with 10 vol equivalents of methanol followed by 10 equivalents of deionized water. This resin was used wet, the amount used being corrected for its 67% water content.

RESULTS AND DISCUSSION

Initial characterization of commercial lipase preparations. Table 2 lists the lipases that were examined, as well as their source organisms. The protein contents of these samples were low and dissimilar. No sample contained more than 40% protein, and several had less than 10%. It has been demonstrated that nonprotein additives can influence the activities and requirements of lipases in organic solvents (22). It is possible that by changing, modifying or removing this material the activities or performance characteristics of the enzymes could be improved.

In aqueous solution, most of the preparations had roughly comparable lipolytic activities, releasing approximately 10 μeq of free fatty acid from olive oil per min per mg solid (Table 2). Exceptions include Lipase G (from *Penicillium cyclopium*), which had one-tenth this activity; calf pregastric lipase, whose activity was too low to measure; and the preparation from *R. javanicus*, whose activity was 13-fold higher than this norm.

The hydrolytic activities of these preparations toward PC in hexane did not parallel their activities toward triacylglycerols in aqueous reactions (Table 2). No activity was observed in the *R. niveus, P. cyclopium* and pregastric preparations. The enzymes from *R. javanicus* and *Pseudomonas* showed moderate activity, and the *C. rugosa, R. delemar* and *H. lanuginosa* samples hydrolyzed PC readily, with activities roughly comparable to that seen earlier for Lipozyme (17). These latter three preparations were selected for further characterization.

Rhizopus delemar lipase specifically hydrolyzes the 1- and 3-position ester bonds of triacylglycerols (23). The *H. lanuginosa* lipase is structurally homologous to this enzyme (24,25) and exhibits the same specificity. Throughout our studies, these enzymes produced 2-lysophosphatidylcholine, which accumulated over the course of PC

TABLE 1

Settings for the Four Experimental Factors Examined

Factor	Minimum		Centerpoint		Maximum
PC[a] (g)	0.1	0.2	0.3	0.4	0.5
Polarity (log *P*)[b] (solvent)	1.3 (2-hexanone)	2.4 (octanone)	3.5 (hexane)	4.5 (isooctane)	5.6 (decane)
Water (μL)					
Humicola lanuginosa	4	22	40	58	76
Candida rugosa	0	32	63	95	126
Rhizopus delemar	0	35	69	104	138
Enzyme (mg)					
H. lanuginosa	2	10	20	30	38
C. rugosa	10	20	30	40	50
R. delemar	10	30	50	70	90

[a]Phosphatidylcholine.
[b]The logarithm of the partition coefficient in an octanol/water two-phase system.

TABLE 2

Propeties of Commercial Lipase Preparations

Manufacturers' designation	Source organism	% Protein	Lipolytic activity[a]	Phospholipolytic activity[b] (mg assayed)
AY	*Candida rugosa*	2.1	16	96% (50)
CE	*Humicola lanuginosa*	20.6	14	86% (50)
D	*Rhizopus delemar*	24.6	12	84% (50)
N	*R. niveus*	10.7	8	0 (100)
FAP-15	*R. javanicus*	38.3	130	40 (100)
G	*Penicillium cyclopium*	3.8	1	0 (100)
PS-30	*Pseudomonas* sp.	4.9	9	27 (100)
PGE	Calf pregastric	7.9	0	0 (100)

[a]Expressed as μeq of fatty acid released from olive oil per min per mg solid enzyme preparation, measured at the optimal pH, which was determined in preliminary experiments.
[b]Expressed as the extent of phosphatidylcholine (PC) hydrolysis achieved during incubation in 8 mL water-saturated hexane containing 0.331 g PC and 75 μL additional water for 15 h at 42°C.

hydrolysis. *Candida rugosa* lipase is a positionally nonspecific enzyme (26). The initial product of its action on PC was also 2-lysophosphatidylcholine. However, this material was further hydrolyzed by *C. rugosa* lipase, resulting in the complete hydrolysis of both fatty acyl esters in PC.

Because it stabilizes the noncovalent interactions that maintain enzyme structure, water plays a critical role in the retention of enzyme activity in organic solvents (27,28). Water is also a substrate in the hydrolytic reaction under investigation here. To obtain PC hydrolysis in hexane, it was necessary to add water over and above that required to saturate the solvent. The choice of water level in this initial scan for activity (Table 2) was guided by our previous experiences with Lipozyme (17). It is possible that the preparations that were inactive (Table 2) would hydrolyze PC at other water levels. However, because at least marginal activity is demonstrated by the active enzymes over wide ranges of water concentration (below), it seems unlikely that this is the case.

It is notable that enzyme preparations from different isolates of *Rhizopus* displayed substantial differences in their abilities to hydrolyze PLs. It has been proposed that these isolates are, in fact, representatives of a single species (29,30). Furthermore, the lipases of *R. delemar* (24) and *R. niveus* (31) have identical amino acid sequences. The activity differences observed here suggest that the individual isolate employed and/or the method of enzyme production can affect the level of activity.

Parameters influencing the activity of the free enzymes. Response-surface methods were adopted to more fully define the effects of solvent and the amounts of water, enzyme and substrate on PC hydrolysis by the three most active enzymes. The resulting estimated regression models are shown in Equations 2–4:

For *H. lanuginosa:*

$$\text{predicted \% hydrolysis} = 37.4 - 5.27A + 7.08B + 1.50C - 16.2D - 0.888AB + 3.25AC + 3.99AD + 0.625BC - 4.53BD - 0.338CD - 3.70A^2 - 3.65B^2 - 5.13C^2 - 0.583D^2 \quad [2]$$

R^2 = coefficient of determination = 0.5803

For *C. rugosa:*

$$\text{predicted \% hydrolysis} = 19.1 - 1.53A + 5.48B - 1.45C + 7.65D - 2.85AB + 4.88AC - 2.68AD - 1.46BC + 2.93BC - 4.33CD - 2.07A^2 + 0.496B^2 - 3.79C^2 - 0.741D^2 \quad [3]$$

$R^2 = 0.6113$

For *R. delemar:*

$$\text{predicted \% hydrolysis} = 13.7 - 8.37A + 4.21B + 4.43C - 13.2D - 2.86AB + 2.89AC + 1.78AD + 1.08BC - 1.601D - 15.1CD + 0.076A^2 + 1.80B^2 - 0.621C^2 + 5.13D^2 \quad [4]$$

$R^2 = 0.7407$

where A = substrate (g), B = enzyme (g), C = water (μL), D = solvent polarity (log P).

Analysis of these equations indicated that, for all three enzymes, solvent polarity had the largest single impact on enzyme activity. The *R. delemar* enzyme was also significantly affected by the cross-product of the polarity and the water concentration. The effects of solvent polarity and the water content of the reaction on the predicted degrees of hydrolysis, calculated from Equations 2–4, are shown in Figure 1.

Each enzyme displayed a different sensitivity to solvent polarity. The predicted activity of the *H. lanuginosa* enzyme increased as the polarity of the solvent increased (Fig. 1A). This behavior was seen at all levels of substrate and enzyme examined, and is similar to that observed for Lipozyme at low and moderate PC concentrations (17).

It has typically been reported that lipases display maximum activity in more nonpolar solvents, often those with log P values greater than 3.5. We initially postulated that the requirement for a relatively polar solvent to achieve optimum PC hydrolysis might relate to the polar nature of the substrate (17). This predicts that all lipases would hydrolyze PC optimally in polar solvents. The results obtained for the *C. rugosa* and *R. delemar* lipases do not fully support this proposal. The *C. rugosa* and *R. delemar* enzymes were qualitatively different from each other, and from *H. lanuginosa*, with regard to the relationship between activity and solvent polarity (Fig. 1). For the *C. rugosa* enzyme, activity in various solvents was

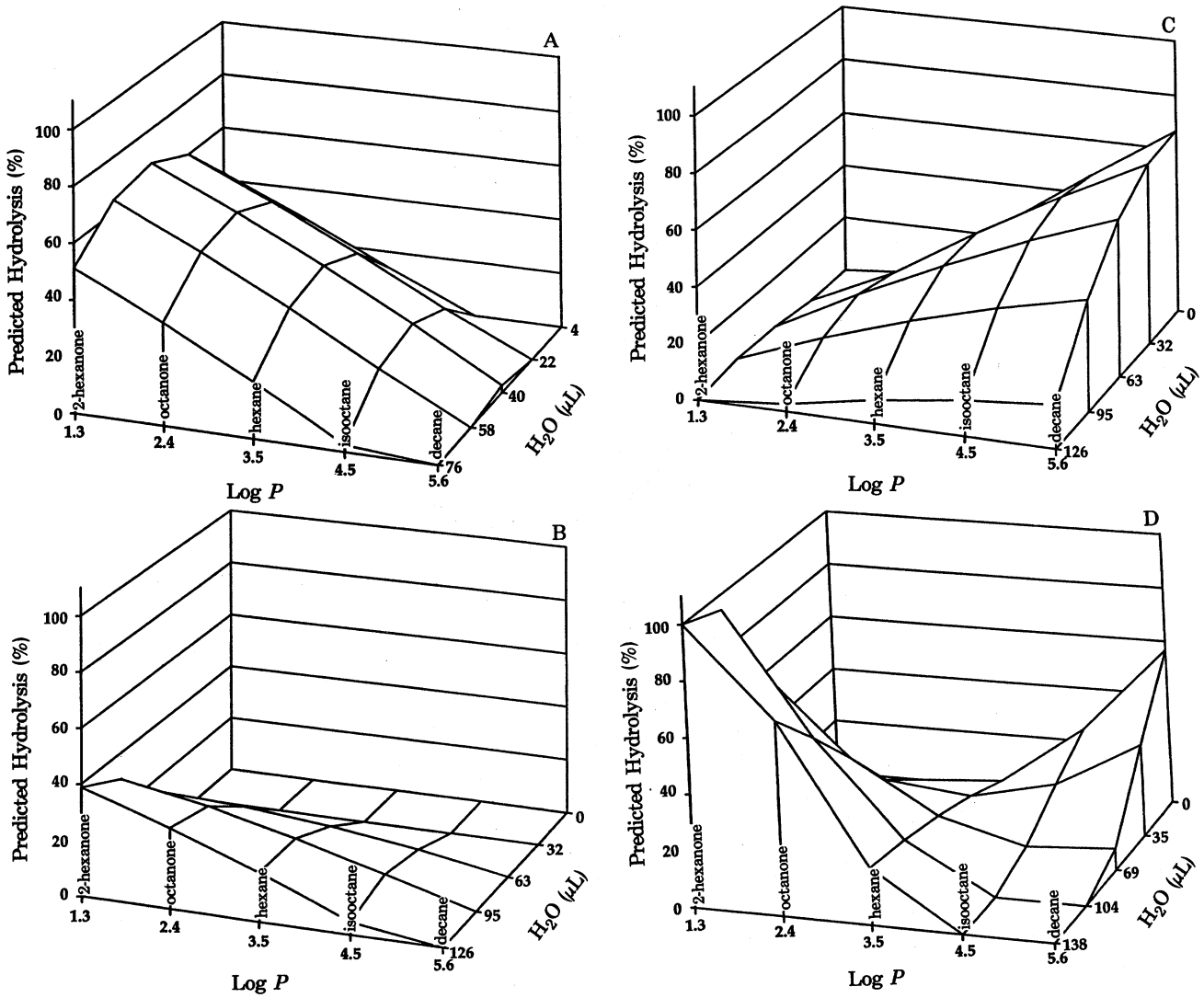

FIG. 1. The predicted degrees of hydrolysis of phosphatidylcholine (PC) by selected commercial lipase preparations as a function of the polarity of the solvent and the amount of water added, based on Equations 2–4. (A) *Humicola lanuginosa* lipase; the amounts of enzyme and PC were the centerpoints of the ranges examined in this series (see Table 1). (B) *Candida rugosa* lipase in reactions containing the minimum amount of enzyme (10 mg) and the maximum amount of substrate (0.5 g). (C) *Candida rugosa* lipase in reactions containing the maximum amount of catalyst (50 mg) and the centerpoint amount of substrate (0.3 g). (D) *Rhizopus delemar* lipase, for reactions where the amounts of catalyst and substrate are at the centerpoints of the ranges examined (see Table 1). Log *P*, the logarithm of the partition coefficient in an octanol/water two-phase system.

modulated by the ratio of substrate to enzyme (Figs. 1B and 1C). At ratios above approximately 20 (mass basis), optimal activity occurred in polar solvents that contained relatively large amounts of added water (Fig. 1B). At ratios below this value, the activity was highest in nonpolar solvents with little additional water (Fig. 1C).

The *R. delemar* lipase displayed a bimodal dependence of activity on solvent polarity throughout the ranges of substrate and catalyst examined here (Fig. 1D). The enzyme was active in both polar and nonpolar solvents, and least active in solvents such as hexane (log $P = 3.5$), whose polarity was in the middle of the range tested. As with the *C. rugosa* enzyme, maximum activity occurred in polar solvents at high amounts of added water, and the activity was highest in nonpolar solvents when no additional water was present (Fig. 1D).

The *H. lanuginosa* and *R. delemar* enzymes belong to a family of 1,3-specific fungal lipases whose members exhibit similarities at the genetic, biochemical and structural levels (Derewenda, U., L. Swenson, Y. Wei, R. Green, P.M. Kobos, R. Joerger, M.J. Haas and Z. Derewenda, submitted for publication; 24,25,32). Substantial differences exist between this family and that to which the *C. rugosa* lipase belongs (33,34). It is possible that structural differences between these groups are the basis of the variations in their requirements for optimal PC hydrolysis. Currently, however, it is not possible to identify the structural basis of these differences or the cause of the variations between the performances of the *H. lanuginosa* and *R. delemar* enzymes. It is clear that, by suitable choice of catalyst, it should be possible to achieve PC hydrolysis under a wide range of reaction conditions.

Effects of immobilization on enzyme activity. Immobilization of an enzyme on a solid carrier simplifies handling,

allows enzyme recovery from reaction mixtures, and may increase the activity or stability of the catalyst. A wide variety of lipases, supports and immobilization conditions have been investigated for use in lipid hydrolysis and interesterification (35). The effects of noncovalent immobilization on the abilities of the *C. rugosa*, *H. lanuginosa* and *R. delemar* lipases to hydrolyze PC under the standard reaction conditions were investigated. In water-saturated hexane, with no additional water, the activities of the immobilized enzymes were low: less than 5% of the PC was hydrolyzed. Upon addition of water, all three immobilized lipases displayed hydrolytic activity (Table 3). No single resin gave uniformly high activity for all enzymes, and no enzyme retained activity to the same extent on all resins. The activity of the *C. rugosa* enzyme was reduced two- to threefold upon immobilization on Celite, polypropylene or Amberlite and was nearly completely eliminated by binding to controlled-pore glass. The activity of the *R. delemar* preparation was doubled by immobilization on Celite and Amberlite and unaffected by binding to glass and polypropylene resins. The *H. lanuginosa* preparation was slightly activated upon binding to Celite and displayed about a twofold reduction in activity on glass and Amberlite.

Factors affecting the activity of Celite-immobilized lipases. Considering the costs of the supports and the activities of the enzymes after immobilization, Celite was chosen as a support in further investigations. The effects of solvent polarity on the activities of the Celite-bound enzymes were explored with hexane, isooctane and octanone (Fig. 2). The amounts of catalyst used in these experiments (*H. lanuginosa*, 30 mg; *C. rugosa*, 60 mg; *R. delemar*, 100 mg) were chosen to obtain linear hydrolytic rates over the course of the 15-h incubations. Immobilization did not greatly affect the relationship between solvent polarity and activity (Fig. 1 vs. Fig. 2). The *R.*

TABLE 3

Phosphatidylcholine (PC) Hydrolysis by Immobilized Lipases

Enzyme source	Support	Relative activity[a]
Candida rugosa	Celite 545[b]	29
	Polypropylene[c]	34
	Controlled-pore glass[d]	3
	Amberlite XAD-7[e]	44
Rhizopus delemar	Celite 545	200
	Polypropylene	105
	Controlled-pore glass	103
	Amberlite XAD-7	226
Humicola lanuginosa	Celite 545	135
	Polypropylene	109
	Controlled-pore glass	43
	Amberlite XAD-7	63

[a]Activities of the lipases following immobilization are expressed relative to those of equivalent amounts of unimmobilized enzyme. The amounts of immobilized lipase assayed were: *H. lanuginosa*, 60 mg; *C. rugosa*, 45 mg; *R. delemar*, 175 mg. Reactions contained 8 mL hexane, 0.331 g PC, and water (40 μL, 63 and 69 μL for the three enzymes, respectively). Incubation temperature was 42°C.
[b]Company source: Fisher Scientific (Fair Lawn, NJ).
[c]Company source: AKZO (Obernburg, Germany).
[d]Company source: Corning BIO systems (Corning NY).
[e]Company source: Sigma Chemical Co. (St. Louis, MO).

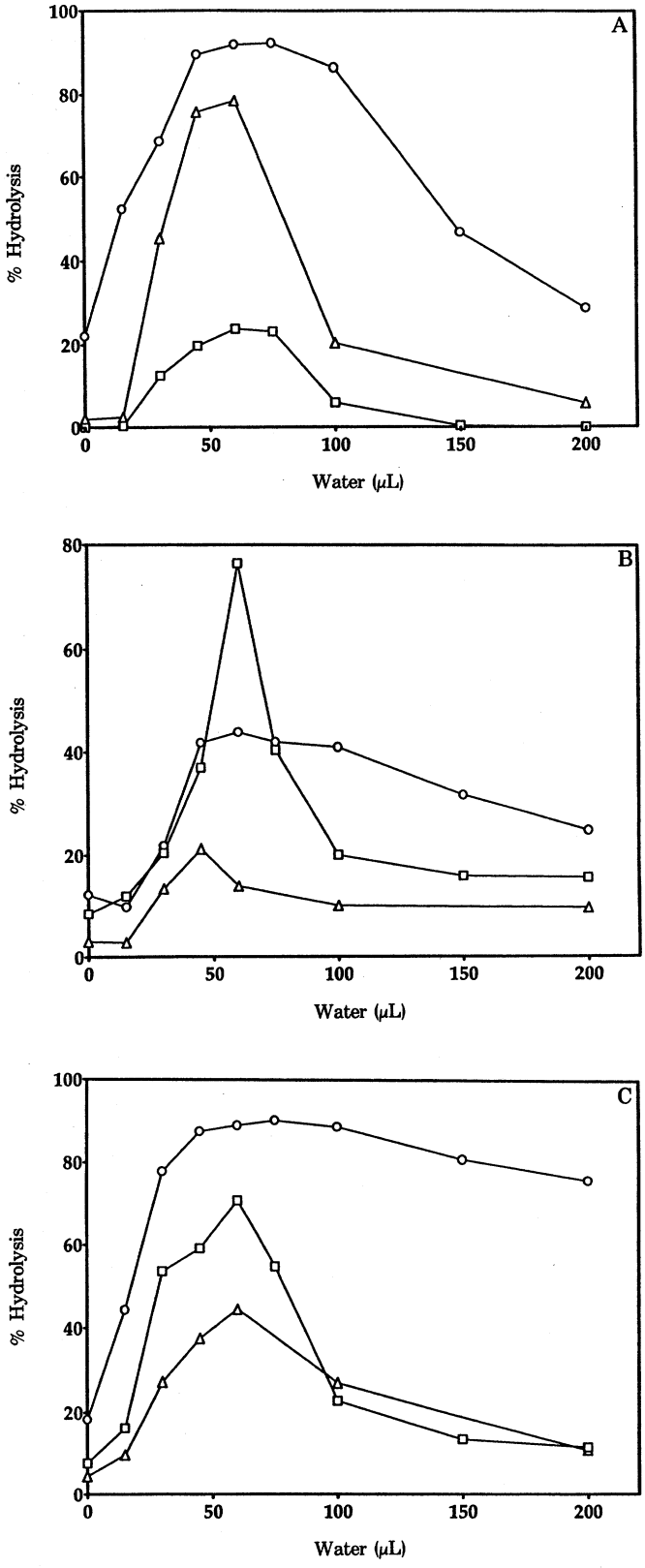

FIG. 2. Relationship of the hydrolytic activities of Celite-immobilized lipases to the choice of solvent and the amount of water over and above that necessary to saturate it. The standard reaction conditions were employed. (A) 30 mg of immobilized *Humicola lanuginosa* lipase; (B) 60 mg of immobilized *Candida rugosa* lipase; (C) 100 mg of immobilized *Rhizopus delemar* lipase. Octanone, ○; hexane, △; isooctane, □.

delemar and *H. lanuginosa* enzymes continued to display maximum activity in octanone, the most polar of the three solvents examined. As seen for the free enzymes, immobilized *R. delemar* lipase was least active in hexane, and the activity of the *H. lanuginosa* preparation decreased as polarity decreased. Immobilization did alter the performance of the *C. rugosa* lipase. The activity of the immobilized enzyme was generally better in octanone and poorer in isooctane than that of the free enzyme.

Although there were small differences in the exact locations of the optima, the three immobilized enzymes displayed maximum activities in all three solvents when between 40 and 70 μL of water were added per 8 mL reaction (Fig. 2). These water contents represent a four- to seven-fold molar excess over that required to completely hydrolyze one of the fatty acyl esters of the PC in the reaction. The *C. rugosa* and *R. delemar* lipases underwent a marked shift in water requirements upon immobilization (Fig. 1 vs. Fig. 2). Evidently, when immobilized on Celite, the enzymes are less sensitive to the effects of solvent polarity on water availability, perhaps indicating that the support helps maintain a suitable amount of water in the vicinity of the enzyme. Valivety *et al.* (36) reported a similar observation in their studies with Lipozyme. For all three enzymes, octanone supported high enzyme activity over a wide range of water levels, while in hexane and isooctane, the ranges of water with best activity were narrower (Fig. 2).

The amount of water required for optimal enzyme activity could be a function of the amount of catalyst present. This would result if the lipases themselves, other proteins, the substantial amounts of nonprotein material in these samples or the carrier affected the availability of water to the enzyme. To test this, standard hydrolytic reactions were conducted with different amounts of immobilized enzyme. The results obtained for *H. lanuginosa* lipase are shown in Figure 3. Despite a sixfold variation in the amount of catalyst, the water level at which maximum hydrolytic activity occurred remained constant at or near 60 μL. Qualitatively similar results were obtained with the immobilized *R. delemar* and *C. rugosa* lipases, with optimum activity occurring at approximately 60 μL of water for the former and 45 μL for the latter. Thus, within the range of enzyme amounts studied here, the volume of water required for optimal activity was independent of the amount of catalyst in the reaction. Figure 3 also shows that the reaction rate was not linearly dependent upon the amount of enzyme in the reaction. There was a higher degree of hydrolysis per mg of catalyst when less enzyme was employed. This suggests the presence of an enzyme inhibitor in one or more components of the reaction mixture.

Due to their polarity, PLs possess a significant water-binding capacity that could affect the availability of water to the catalyst. The ability of the substrate concentration to modulate the amount of water required for hydrolysis was therefore investigated. The results obtained with immobilized *H. lanuginosa* lipase (30 mg) are typical of those also obtained for the other two imobilized preparations when each was incubated in hexane containing various amounts of PC and water (Fig. 4). As the concentration of substrate increased, the water level that conferred maximum hydrolytic activity increased proportionately. For all three enzymes, the optimal activity occurred at a molar ratio of added water to PC of between 5 and 8. Thus, substrate concentration played a prominent role in determining the water requirements of the immobilized enzymes. In their studies of Lipozyme-mediated esterification in organic solvents, Valivety *et al.* (36) similarly found that the substrate can greatly increase the water capacity of the solvent in which it is dissolved. This effect is particularly important in the case of polar substrates, such as PLs, which have appreciable water affinities. Enzyme activity will be reduced or eliminated if these effects are not compensated by the addition of an appropriate amount of water. This can be achieved by adding water at the start of the reaction, as was done here. However, the method of Kvittingen *et al.* (37), in which salt hydrates

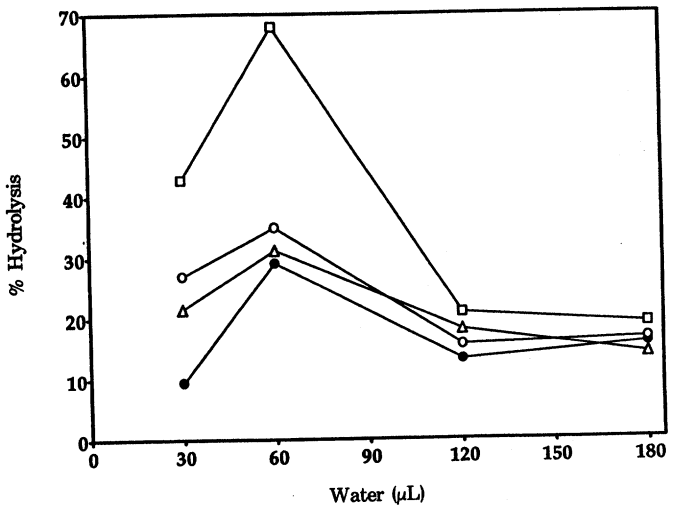

FIG. 3. The hydrolysis of phosphatidylcholine by Celite-immobilized *Humicola lanuginosa* lipase as a function of enzyme and water concentrations in the standard reaction. Incubation time: 3 h (●), 30 mg catalyst; (△), 60 mg; (○), 90 mg; (□), 180 mg.

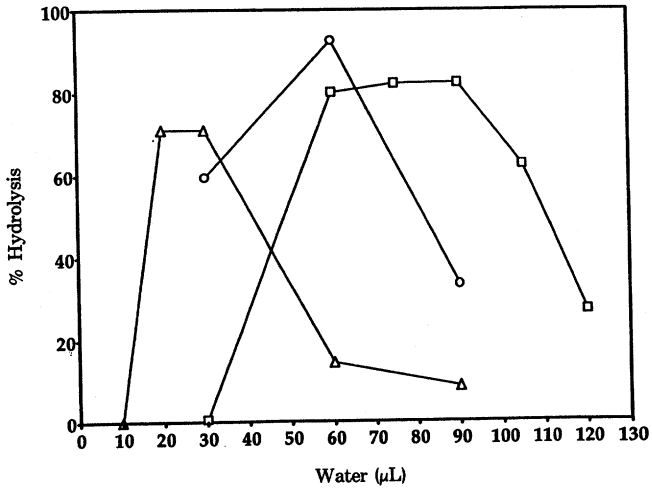

FIG. 4. Effect of substrate concentration on the water requirements for phosphatidylcholine (PC) hydrolysis by 30 mg of immobilized *Humicola lanuginosa* lipase. Standard reactions were conducted at 42°C in 8 mL of water-saturated hexane containing 30 mg of enzyme and either 167 (△), 331 (○) or 497 (□) mg of PC.

are employed to regulate the water content of the reaction, could be a superior method of compensating for changes in water capacity that occur either from run to run or over the course of a reaction.

Lipase activities in organic solvents are known to be sensitive to the water content of the system. Previous studies, primarily of lipase-catalyzed esterification, have demonstrated that, in completely anhydrous systems, the enzymes display little or no activity. As water content is increased, the enzymes become active, not only because water is a co-reactant in the hydrolytic reaction but also presumably because it stabilizes essential bonds and promotes the molecular flexibility required for catalysis. Further water addition increases activity, up to a maximum, beyond which it declines. This reduction is associated with clumping of the catalyst particles, which reduces interfacial area and limits mass transfer. The relationship between enzyme activity and water content is usually solvent-dependent. Halling and co-workers (36–38) have unified a diversity of observations in this area with their demonstration that reaction rate is responsive, not to the absolute amount of water in the system but to the thermodynamic water activity of the reaction mixture. This parameter is affected by the water-binding capacities of all components of the reaction mixture, including catalyst, reactants and products. The dependence of activity on water content, which we report here, is analogous to that seen for esterification reactions and emphasizes the fact that these enzymes require water activities greater than the stoichiometric requirement of the reaction. This explains our observations that: (i) mere saturation of the solvents with water, prior to the addition of substrate and enzyme, is insufficient to give hydrolysis; and (ii) the amount of additional water required is a function of the PL concentration. PLs are sufficiently polar that they bind water strongly in organic solvents. For example, in the studies here, the addition of as much as 65 μL of water over and above that necessary to saturate the 8 mL of hexane in the standard reaction resulted in clear, single-phase solutions. This binding must appreciably reduce the water activity of the system, even at the relatively low PC concentration (70 mM) used in the standard reaction. These observations emphasize the degree to which the substrate can moderate the availability of water to the catalyst during enzymatic hydrolysis in organic solvents.

Factors affecting enzyme reuse. The ability to retain activity in consecutive reactions plays a major role in determining the practical utility of a biological catalyst. The factors affecting enzyme activity in repeated PC hydrolysis reactions were investigated for Lipozyme, a versatile immobilized lipase (39–41). When 150 mg of Lipozyme was incubated in a standard reaction containing 60 μL of water, the optimum for this enzyme (17), an average of 75% of the substrate was hydrolyzed in a 12-h incubation at 42°C. However, reuse of the Lipozyme in a second batch reaction resulted in less than 10% hydrolysis. Preliminary experiments indicated that the water requirements of the enzyme were altered after the first reaction. To investigate this, six standard reactions were conducted with 150 mg Lipozyme and 60 μL water. Each liquid phase was then replaced by fresh reactant solution that contained one of six different amounts of water, and incubation was repeated. Four such repeat cycles were conducted. The degree of hydrolysis in each is shown in Figure 5. The highest retained activity at the final cycle (77% of initial activity) was achieved with 30 μL of water. With either more or less water, the hydrolytic activity was reduced, in most cases to zero, by the fifth incubation. The poorest performance was obtained in reactions containing 60 μL of water, the amount that gives highest activity in a single-reaction format. Under the conditions giving optimal activity retention, the molar ratio of added water to PC was 3, similar to the ratio that produced optimal activity for the Celite-immobilized enzymes over a range of substrate concentrations (Fig. 4). The requirement for a higher level of water to achieve maximum activity in the initial reaction suggests that the hydration state of the catalyst undergoes adjustment early in the reaction, after which the reaction proceeds with lower water inputs in subsequent cycles.

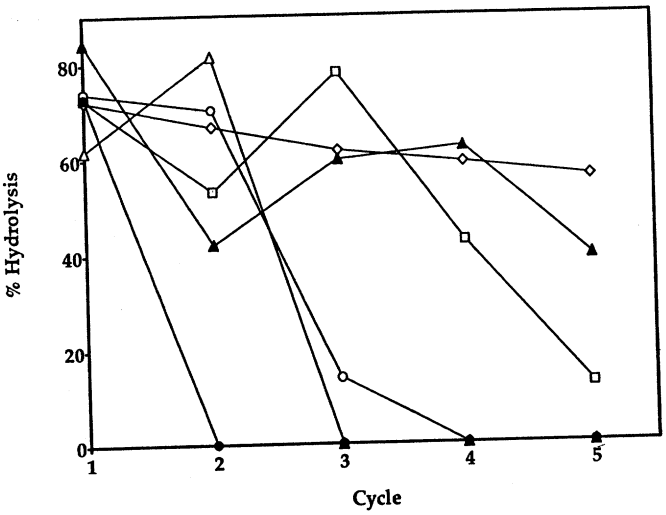

FIG. 5. The effect of the water content of the reaction on the retention of activity by Lipozyme IM20 (NOVO Biolabs, Danbury, CT) in successive batch reactions of phosphatidylcholine-hydrolysis in hexane. In the first cycle, 0.15 g of Lipozyme was incubated under the standard reaction conditions for 12 h in the presence of 60 μL of added water. In subsequent cycles, the liquid phase was replaced by fresh reactants containing the indicated amounts of water [0 μL (△), 7.5 μL (○), 15 μL (□), 30 μL (◇), 45 μ (△), 60 μL (●)], and incubation was repeated.

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